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Effects of Subchronic Inhalation of Low Concentrations of Nitrogen Dioxide. 1 The Proximal Alveolar Region of Juvenile and Adult Rats

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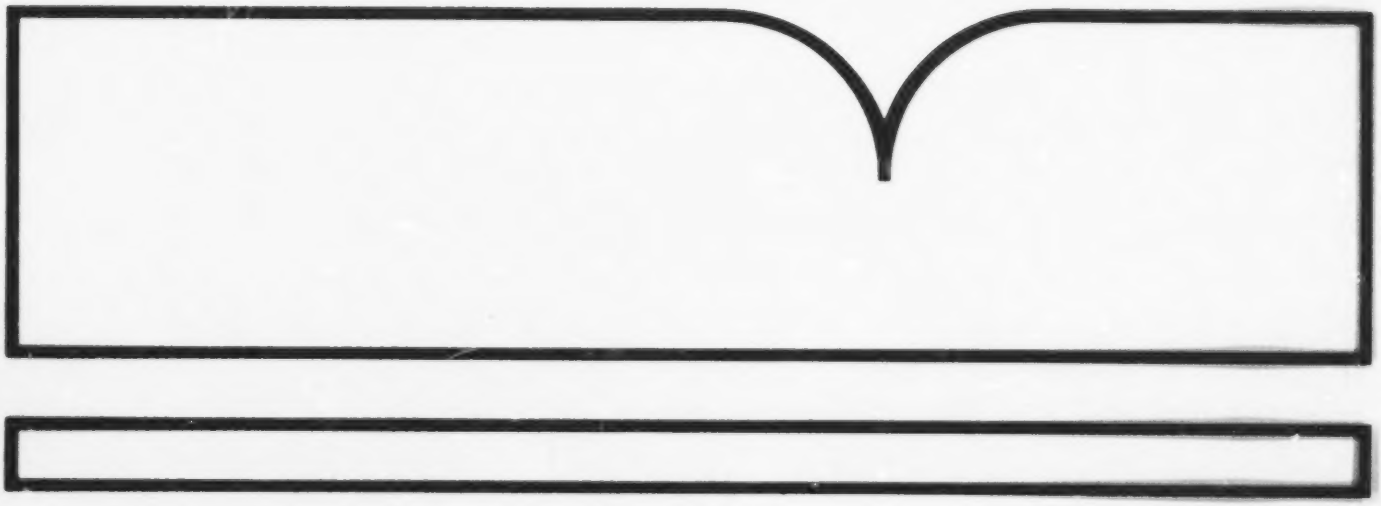

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16. ABSTRACT

Techniques were devised to isolate tissue from the epithelium of terminal airways and the alveoli proximal to the airways. One day old juveniles and six week old adult rats were exposed to either room air or 0.5 ppm NO_2 for 23 hrs per day seven days per week. An additional group of adult rats were exposed to 2.0 ppm NO_2 for the same duration. Two daily hour spikes to three times the background levels (0.5 to 1.5 ppm and 2.0 to 6.0 ppm) were applied Mon. through Fri. Morphometric analysis revealed that epithelial injury occrred in all exposed anaimals. The juvenile rats which had been exposed to 0.5 ppm NO_2 exhibited changes in the characteristics of type II epithelial cells. These cells spread to cover more alveolar surface and became thinner. Adult animals exposed to 0.5 and 2.0 ppm NO_2 showed changes in alveolar macrophages and in the alveolar interstitium in addition to changes in the epithelium. Animals exposed to 0.5 ppm NO_2 showed spreading and hypertrophy of type II cells. Those animals exposed to the higher concentration of NO_2 had similar changes in type II cells and in addition showed an increase in type I cell number. The type I epithelial cells were smaller and covered less alveolar surface area than normal type I cells. Adult rats were as sensitive or more sensitive to NO_2 injury than were juvenile rats.

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Effects of Subchronic Inhalation of Low Concentrations of Nitrogen Dioxide

I. The Proximal Alveolar Region of Juvenile and Adult Rats[1]

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Effects of Subchronic Inhalation of Low Concentrations of Nitrogen Dioxide. I. The Proximal Alveolar Region of Juvenile and Adult Rats. CHANG, L.-Y., GRAHAM, J. A., MILLER, F. J., OSPITAL, J. J., AND CRAPO, J. D. (1986). *Toxicol. Appl. Pharmacol.* **83,** 46–61. Inhalation of nitrogen dioxide (NO_2) produces injury to the epithelium of terminal airways and the alveoli proximal to the airways. Techniques were devised to isolate alveolar tissue from this region for morphometric studies to define the extent of alveolar septal injury caused by NO_2. One-day-old and six-week-old rats were exposed to either room air or 0.5 ppm NO_2 for 23 hr per day 7 days per week for 6 weeks. An additional group of 6-week-old rats were exposed to 2.0 ppm NO_2 for the same duration. Two daily hour spikes to three times the background concentrations (0.5 to 1.5 ppm and 2.0 to 6.0 ppm) were applied Monday through Friday. At the end of the exposure, rat lungs were fixed by intratracheally infusing buffered 2% glutaraldehyde. Pieces of lung tissue were embedded in large plastic blocks which were softened with heat and thin (0.3 mm) sliced. Terminal bronchioles and their corresponding proximal alveolar regions were identified from the thin plastic slices, removed, and glued to cylindrical EM blocks for thin sectioning. Morphometric analysis revealed that epithelial injury occurred in all exposed animals. The juvenile rats which had been exposed to 0.5 ppm NO_2 since 1 day of age exhibited changes in the characteristics of type II epithelial cells. These cells spread to cover more alveolar surface and became thinner. Adult animals exposed to 0.5 and 2.0 ppm NO_2 showed changes in alveolar macrophages and in the alveolar interstitium in addition to changes in the epithelium. Animals exposed to 0.5 ppm NO_2 showed spreading and hypertrophy of type II epithelial cells. Those animals exposed to the higher concentration of NO_2 had similar changes in type II epithelial cells and in addition showed an increase in type I cell number. The type I epithelial cells were smaller and covered less alveolar surface area than normal type I cells, suggesting a regenerating population of type I cells. These results suggest that prolonged exposure to low concentrations of NO_2 can cause injury to the alveolar epithelium indicated initially by spreading and hypertrophy of type II cells followed by differentiation into type I cells to compensate and repair the injury. Adult rats were as sensitive or more sensitive to NO_2 injury than were juvenile rats. © 1986 Academic Press, Inc.

NO_2 is a major pollutant of urban environments. Its major source is the combustion of fossil fuels. The current U.S. National Ambient Air Quality Standard for NO_2 is 0.05 ppm (100 $\mu g/m^3$) for the annual arithmetic mean of 24-hr values. Average 24-hr NO_2 concentrations in urban atmospheres vary widely but in some cities can commonly reach the range of 0.05 to 0.2 ppm. Some urban communities report elevated NO_2 concentrations (spikes) as high as 0.5 ppm (CARB,

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1983). In view of the adverse health effects NO_2 may cause (Dawson and Schenker, 1979), it is essential to determine the effects of chronic exposure to near ambient concentrations of NO_2 to develop appropriate environmental controls. Chronic exposure to high concentrations of NO_2 can induce emphysema-like changes in rat lungs (Freeman and Haydon, 1964; Freeman *et al.*, 1968). Most of the past studies concerning near ambient concentrations of NO_2 have involved qualitative assessments of cell injuries. Rigorous quantitative data on the effects of near atmospheric concentrations of NO_2 on the respiratory system are needed. The current study was undertaken as a first step toward obtaining such information. The primary goal of this study was to investigate the effects of prolonged exposure to low concentrations of NO_2 by morphometric methods designed to define the extent of specific structural alterations.

Previous investigations on the toxicity of NO_2 have established that the primary areas of lung injury after exposure are the terminal airways (Stephens *et al.*, 1971; Parkinson and Stephens, 1973) and the alveoli and alveolar ducts adjacent to terminal airways (Stephens *et al.*, 1972). Ciliated cells and type I epithelial cells have been shown to be susceptible to injury by NO_2. Loss of cilia from ciliated cells and vesiculation and membrane blebbing of type I epithelium have been observed after exposure to 1 to 2 ppm NO_2. This report focuses on the effects of exposure to 0.5 and 2.0 ppm NO_2 in the proximal alveolar region. This region is defined as including septa immediately adjacent to terminal or respiratory bronchioles. Methods were devised to reproducibly isolate this specific region for ultrastructure studies. Morphometric techniques were applied to quantify tissue changes and characterize cell structural alterations caused by the NO_2 exposures.

METHODS

Animals and exposure conditions. One-day or 6-week-old, male, Fisher 344 rats (CDF, F-344 Crl BR, Charles River Laboratories, Wilmington, Mass.) were used for the experiments. The rats were exposed to either room air or NO_2 for 6 weeks in stainless-steel Rochester type chambers having a volume of 0.323 m^3. At the end of the exposure the rats were either 6 or 12 weeks old and were termed juvenile and adult rats, respectively. The adult rats were weight matched at the beginning of the exposure. One-day-old juvenile rats were obtained from timed pregnant female rats housed in the exposure chambers beginning 1 week prior to delivery while exposed to filtered room air. Shredded Teflon was used as a nesting material. Six male pups were selected from each litter within 24 hr of birth. Male pups from 2 to 3 litters were combined to achieve a complement of 6 when the number of males in a single litter fell short. Exposure was initiated on the morning following birth. Mothers were kept in the cages with the pups for the first 3 weeks of the exposure, after which the pups were weaned onto Purina Rat Chow.

Groups of juvenile and adult rats were exposed to 0.5 $\pm$ 0.005 ($\pm$SD) ppm NO_2 for 23 hr a day, 7 days a week for 6 weeks. Two 1-hr spikes to 1.5 ppm were applied at 8:00 in the morning and 4:00 in the afternoon. The exposure was interrupted for 1 hr each day between noon and 1:00 pm for maintenance of the animals. An additional group of adult rats were exposed to 2.0 $\pm$ 0.02 ppm NO_2 with two daily spikes to 6 ppm with the same timing. The twice a day 1-hr spikes were instituted to simulate the diurnal spikes in ambient NO_2 concentrations that occur in some urban environments. For ease of discussion, these exposure regimens will be henceforth referred to by the baseline NO_2 concentrations.

During the exposures, rats were housed individually (except for unweaned pups). Stainless-steel hanging wire mesh cages were used. Nitrogen dioxide was supplied as a mixture of 1% NO_2 in air from bottled gas tanks to the air flowing through the chambers which was maintained at a constant flow of 323 liter/min. The chamber concentration of NO_2 was monitored by continuous chemiluminescent analyzers (Bendix 8101C) (EPA 1975). The nitrogen dioxide analyzers were calibrated every 2 weeks and were audited quarterly by an external contractor. The delivery of NO_2 to the chambers was regulated by mass flow controllers which could be controlled either automatically or manually. Control rats were maintained in the same type of chambers but exposed only to filtered room air. Juvenile rats weighed 4–6 g at birth and had an average weight of 125–140 g at the end of the 6-week exposure. Adult rats weighed 120–135 g at 6 weeks of age and reached an average of 257–263 g at the end of the experiment. All animals received feed and water *ad libitum*.

Preparation of tissue. Rats were anesthetized by an ip injection of pentobarbital before their tracheas were cannulated. The abdominal cavity was opened and both hemidiaphragms were punctured to deflate the lungs. The lungs were then fixed by inflation with 2% glutaraldehyde in 0.085 M Na cacodylate buffer (Crapo *et al.*, 1980), at a constant pressure of 20 cm above the midpoint of the chest wall. Four 2-mm thick transverse slices, two each

from the upper and the lower one-third of the left lung, were taken. Each slice was further cut into 3 to 4 pieces. These tissue pieces were washed in cacodylate buffer, post-fixed with osmium tetraoxide, dehydrated in a graded series of alcohol, and then transferred to propylene oxide. Because of the size of the blocks, care was taken to use large amounts of fluid (>10 times the tissue volume) for each alcohol and propylene oxide change. The dehydrated tissue pieces were taken through three 1-hr changes of graded plastic-propylene oxide mixtures (1:2, 1:1, and 2:1), rinsed with degased 100% plastic, and then left in 100% plastic overnight. Lung tissue pieces were transferred to embedding molds with 4 × 4 × 6 mm troughs filled with fresh resin and cured at 60°C for 16 to 24 hr. The plastic used for this embedding procedure was Epox 812 (Ernest F. Fullem, Inc.) with an A (Epox & DDSA) to B (Epox & NMA) ratio of 7 to 3.

Isolation of proximal alveolar region. The proximal alveolar region was isolated by a modification of the technique originally described by Barry *et al.* (1985). The large Epox blocks were first softened with mild heat (40°C) and then cut into slices approximately 0.3 mm thick. Each slice was examined with a dissecting microscope. Through random chance, some slices contained terminal bronchioles that were cut in a direct cross-section and which lead into the proximal alveolar region as demonstrated by visualizing first bifurcation of the alveolar duct. Four such specimens, from four different blocks, were selected from each animal. A 1-mm block containing this region was cut from each slice and glued to blank EM blocks for ultramicrotomy. Terminal bronchiolar tissue was shaved away with a razor blade. Thick (1-μm) serial sections were then cut from the block until the first alveolar duct bifurcation was reached (Fig. 1). Excess tissue was trimmed away so that only tissue surrounding the first bifurcation and the alveolar ducts remained. Thin sections (60–90 nm) were then cut, picked up on 200 mesh grids, and stained with uranyl acetate and lead nitrate.

Morphometric methods. The thin sections of the proximal alveolar region usually covered 15–20 grid squares. Two electron micrographs at a magnification of 2000× were taken from each grid square, one from the upper left and one from the lower left corner. Micrographs were printed at 8500× on 11 × 14-in. photographic paper. A counting overlay consisting of 112 lines, each 2 cm long, was superimposed on each micrograph. Point and intercept counting were done to determine volume and surface densities. Numerical density (N_V) was derived by the formula $N_V = N_A/\bar{D}$. Mean caliper diameter values, $\bar{D}$, for the alveolar cells were taken from data on normal rats as reported by Pinkerton *et al.* (1982). The number of nuclear profiles per unit area, N_A, was counted directly on the electron microscope. Fibroblasts and macrophages in the interstitium were separately identified by the criteria described by Barry *et al.* (1985).

The formulae used in the morphometric analysis have been described in detail (Barry and Crapo, 1985; Barry *et al.*, 1985; Weibel, 1979). The margins of the proximal

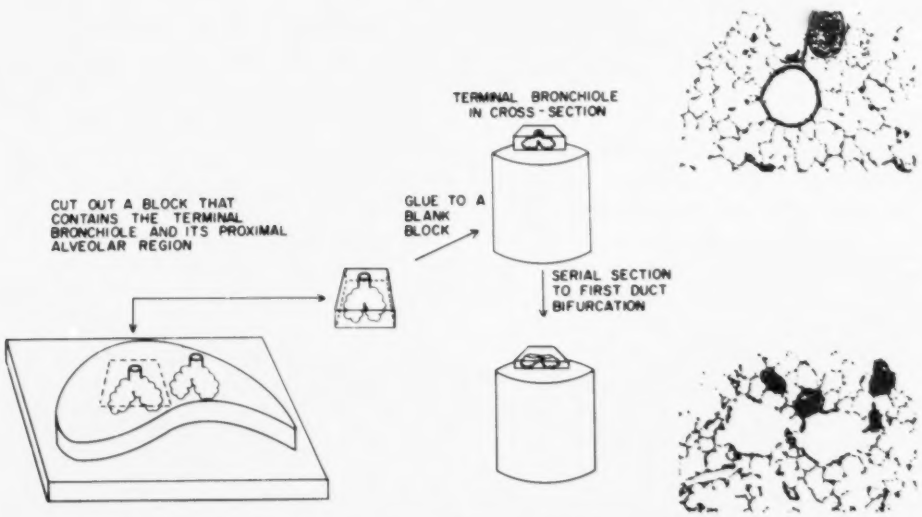


FIG. 1. Method used to isolate proximal alveolar regions from rat tissue slices embedded in epoxy resin. A piece of embedded lung tissue was sliced with a razor blade. Terminal bronchioles that lead into the alveolar region were microdissected and glued to a cylindrical EM blank block for thin sectioning. Serial sections on this oriented block were taken until the first alveolar duct bifurcation was transected.

alveolar region are difficult to define rigorously. Because of this difficulty, volume, surface, and numerical densities cannot be easily expressed as absolute values. To compare data from the various groups in an unbiased manner, volume (V_v), surface (S_v), and numerical densities were divided by the surface density of alveolar epithelial basement membrane (S_vBM) in the same sample. This process normalized all data to the basement membrane surface area. Variance that might result from different degrees of lung inflation would be reduced by this normalization procedure.

Statistical analysis. Data from the two groups of juvenile rats (control and 0.5 ppm NO_2) were compared by Student's *t* test. All comparisons were two-sided and a *p* value less than 0.05 was considered significant. Data from the 3 groups (control, 0.5 ppm, and 2.0 ppm NO_2) of adult rats were analyzed by analysis of variance and Duncan's multiple range test. The effect of age on gas exposures was examined by applying one-way analysis of variance in combination with appropriately selected single degree of freedom contrasts to the data from both the juvenile and adult rats, excluding the 2.0 ppm NO_2-exposed adult rats. The overall contrast for gas × age tests the hypothesis that there is an age-dependent response to the NO_2 exposure, or, in other words, that the adult animals show a greater reaction to NO_2 than do the juvenile animals.

RESULTS

Comparison of the Effects of 0.5 ppm Nitrogen Dioxide on Juvenile and Adult Rats

The results are summarized in Tables 1 and 2. Exposure to 0.5 ppm NO_2 did not cause a change in the body weights of either juvenile or adult rats. Likewise, the lung volumes of both groups were not affected. The surface density of the alveolar basement membrane of exposed, juvenile rats was identical with that of their matched controls. Adult rats exposed to the same concentration, however, showed a 30% increase in the surface density of alveolar basement membrane over the control value. The volume of air in the proximal alveolar regions of 0.5 ppm NO_2-exposed adult rats decreased correspondingly.

An age effect was demonstrated in most of the parameters in Tables 1 and 2. These differences reflect mainly the smaller size of the juvenile lungs and their earlier developmental stage. Exposure to 0.5 ppm NO_2 had few effects on the juvenile animals. None of the tissue volumes in the alveolar septum were altered after the exposure to 0.5 ppm NO_2. In contrast, a number of changes occurred in similarly exposed adult rats. The total volume of alveolar tissue increased 24%. This was due to increases in the volume of type II epithelium (108%), fibroblasts (23%), interstitial matrix (20%), and alveolar macrophages (130%). No effect could be measured on type I epithelium. Few macrophages were found in the interstitium, and there was no increase in interstitial macrophages after exposure to 0.5 ppm NO_2.

The characteristics of type I epithelial cells in both juvenile and adult rats were not changed by the gas exposure (Fig. 2A). Qualitative morphologic evaluation of the alveolar surface epithelium showed occasional small areas of type I cell membrane blebbing (Fig. 3A). This could be found in at least one area on about half of the sections of proximal alveolar region studied from adult animals exposed to 0.5 ppm NO_2. It was found to a lesser extent in juvenile rats and was never found in control animals. Abnormal type I cells with clear cytoplasm were also found in the adult animals (Fig. 3B).

Type II epithelial cells in both the juvenile and the adult rats were reactive, showing changes both in cell volume and in mean cell surface area (Fig. 2B). The relative number of type II cells in the proximal alveolar region was not affected by the gas exposure in both cases. The mean cell alveolar surface or average air border of type II epithelial cells became 47 and 44% larger in juvenile and adult rats, respectively, after exposure. While the mean size of type II cells was only slightly increased (16%) in the juvenile rats, it was 79% larger in adult rats exposed to NO_2. The mean cell thickness of the type II cells decreased in exposed juvenile rats but increased, nonsignificantly, in treated adult animals. The pattern of response in juvenile and adult rats after 0.5 ppm NO_2 exposures was significantly different with respect to changes in type II cell size and thickness. Figure 4 compares normal type II cells to those commonly found in exposed juvenile and adult rats. Spreading of

TABLE 1

ABSOLUTE VOLUMES, SURFACE AREAS, AND DIFFUSION CAPACITY FOR PROXIMAL ALVEOLAR REGION OF THE LUNG

	Juvenile rats		Adult rats			Overall contrasts		
n	Controls (8)	0.5 ppm NO_2 (8)	Controls (12)	0.5 ppm NO_2 (8)	2 ppm NO_2 (8)	Gas	Age	Gas × age
Body weight	130 ± 5	136 ± 5	255 ± 2	259 ± 1	260 ± 2			
Total lung volume (μm^3)	5.0 ± 0.2	4.8 ± 0.2	7.6 ± 0.2	8.1 ± 0.2	8.3 ± 0.2	–	++	++
Basement membrane surface density (S_VBM) ($\mu m^2/\mu m^3$)	540 ± 24	541 ± 39	567 ± 19	739 ± 19*	647 ± 40†	++	++	++
Volumes (V_V/S_VBM) (μm)								
Air	17.10 ± 0.75	17.27 ± 1.16	15.79 ± 0.54	11.36 ± 0.41*	13.62 ± 1.10	+	++	++
Capillary	0.727 ± 0.091	0.823 ± 0.069	1.118 ± 0.075	1.101 ± 0.042	1.219 ± 0.038	–	++	–
Tissue	1.105 ± 0.073	1.088 ± 0.065	0.917 ± 0.030	1.136 ± 0.028*	1.076 ± 0.034*	+	–	+
Type I epithelium	0.214 ± 0.016	0.216 ± 0.010	0.198 ± 0.048	0.215 ± 0.085	0.241 ± 0.074*,†	–	–	–
Type II epithelium	0.076 ± 0.009	0.099 ± 0.010	0.063 ± 0.005	0.131 ± 0.004*	0.102 ± 0.009*,†	++	–	++
Fibroblasts	0.293 ± 0.017	0.260 ± 0.016	0.167 ± 0.009	0.205 ± 0.007*	0.182 ± 0.008	–	++	++
Interstitital macrophages	0.002 ± 0.001	0.003 ± 0.002	0.001 ± 0.001	0.004 ± 0.001	0.003 ± 0.001	–	–	–
Interstitial matrix	0.159 ± 0.010	0.155 ± 0.014	0.217 ± 0.010	0.260 ± 0.013*	0.252 ± 0.012	–	++	±
Endothelium	0.326 ± 0.023	0.315 ± 0.025	0.240 ± 0.070	0.263 ± 0.056	0.251 ± 0.011	–	++	–
Alveolar macrophages	0.032 ± 0.008	0.036 ± 0.007	0.023 ± 0.004	0.053 ± 0.005*	0.041 ± 0.004*	+	–	±
Surface area**								
% Type I	97.4 ± 0.5	95.9 ± 0.4*	98.0 ± 0.1	96.7 ± 0.2*	97.2 ± 0.2*	++	+	–
% Type II	2.6 ± 0.5	4.0 ± 0.4*	1.9 ± 0.1	3.1 ± 0.2*	2.7 ± 0.2*	–	–	–
Capillary epithelium	98.9 ± 3.1	100.9 ± 1.8	89.1 ± 1.3	93.5 ± 3.3*	92.5 ± 1.6	±	++	–
D_T ($\mu l\ O_2 \cdot min^{-1} \cdot mm\ Hg^{-1} \cdot cm^{-2}$)	0.977 ± 0.051	0.920 ± 0.024	0.866 ± 0.022	0.810 ± 0.029	0.799 ± 0.026	–	++	–

Tissue thickness (μm)								
Harmonic mean (air to plasma)	0.342 ± 0.019	0.362 ± 0.011	0.363 ± 0.009	0.398 ± 0.001	0.400 ± 0.001*	−	+	−
Arithmetic mean								
Epithelium	0.293 ± 0.023	0.318 ± 0.020	0.267 ± 0.008	0.351 ± 0.010*	0.347 ± 0.001*	++	−	+
Type I	0.220 ± 0.017	0.226 ± 0.011	0.202 ± 0.005	0.222 ± 0.009	0.248 ± 0.008*†	−	−	−
Type II	3.539 ± 0.479	2.490 ± 0.134*	3.488 ± 0.264	4.362 ± 0.306	3.832 ± 0.228	−	+	++
Interstitium	0.455 ± 0.024	0.417 ± 0.028	0.410 ± 0.017	0.485 ± 0.020*	0.454 ± 0.020	−	−	+
Endothelium	0.332 ± 0.025	0.311 ± 0.022	0.271 ± 0.010	0.281 ± 0.007	0.272 ± 0.012	−	+	−

Note. All data are $\bar{x} \pm$ SE. For the adult animals, comparisons among 3 groups were done by a multiple comparison procedure. *$p < 0.05$ for comparison to their respective control group. †$p < 0.05$ for comparison to adult animals exposed to 0.5 ppm NO_2. Overall contrasts for significant effects due to age and/or to exposure to NO_2 include only those groups exposed to air or 0.5 ppm NO_2. ++$p < 0.01$, +$p < 0.05$, ±$p < 0.10$, −$p > 0.10$ for two-sided tests. **Type I SA is the SA of basement membrane under type I cells; type II SA is the air surface of type II cells excluding the extra SA contributed by microvilli. Endothelial SA is the luminal surface of the endothelial cells expressed as a percentage of the alveolar basement membrane SA. D_T is the tissue component of pulmonary diffusion capacity expressed per cm^2 of epithelial basement membrane.

type II epithelial cells, common in both juvenile and adult NO_2-exposed rats, resulted in significant increases of the fraction of alveolar surface area covered by type II cells and this was accompanied by significant decreases in the fraction of alveolar surface covered by type I epithelial cells. No changes other than those associated with type II epithelial cells were observed in exposed juvenile rats. It was concluded that age has a definite influence on the effect of NO_2 exposure on rat lungs as indicated by the numerous significant differences identified in the column "gas × age" in Tables 1 and 2.

Comparison of the Effects of 0.5 and 2.0 ppm NO_2 on Adult Rats

Morphometric measurements of tissue volume, tissue thickness, and alveolar surface distributions on the three adult rat exposure groups (air, 0.5 ppm NO_2, and 2.0 ppm NO_2) are listed in Table 1. Alveolar cell characteristics from the same three groups of rats are listed in Table 2.

Body weights and fixed lung volumes were not affected by exposure to either concentration of NO_2. Exposure to 0.5 or 2.0 ppm NO_2 caused similar increases in the total tissue volume. The volume of air in the proximal alveolar region decreased after exposure to 0.5 ppm NO_2 but was unchanged by 2.0 ppm NO_2. The volume of capillaries was not affected by either exposure. Both concentrations of NO_2 caused the volumes of alveolar macrophages and epithelium to increase significantly. The epithelial response associated with 0.5 ppm NO_2 was limited to type II epithelial cells, while the response elicited by 2.0 ppm NO_2 involved both type I and type II epithelial cells. Exposure to 0.5 ppm, but not 2.0 ppm, NO_2 caused an increase in the volumes of fibroblasts and interstitial matrix when compared with control values. These changes in tissue volumes did not have a statistically significant effect on the tissue component of diffusion capacity (D_T) (Weibel, 1979) of the alveolar septa in the proximal alveolar region.

TABLE 2

MORPHOMETRICALLY DETERMINED CHARACTERISTICS OF CELLS IN THE PROXIMAL ALVEOLAR REGION OF THE LUNG

	Juvenile rats		Adult rats			Overall contrasts		
n	Controls (8)	0.5 ppm NO_2 (8)	Controls (12)	0.5 ppm NO_2 (8)	2 ppm NO_2 (8)	Gas	Age	Gas × age
Total number of cells (/mm^2)	2480 ± 109	2530 ± 158	1168 ± 55	1195 ± 24	1110 ± 40	−	++	++
Alveolar type I cells								
Number (/mm^2)	129 ± 14	123 ± 14	99 ± 4	93 ± 6	116 ± 5*†	−	+	−
Average volume (μm^3)	1817 ± 241	1877 ± 185	2037 ± 97	2379 ± 169	2111 ± 134	−	−	−
Average surface area (μm^2)	8279 ± 991	8349 ± 767	10447 ± 562	11330 ± 831	8807 ± 416*†	−	+	−
Average basement membrane SA/Cell (μm^2)	8633 ± 1035	8715 ± 830	10116 ± 526	10791 ± 807	8463 ± 369*†	−	+	−
Alveolar type II cells								
Number (/mm^2)	288 ± 23	327 ± 31	141 ± 9	162 ± 5	155 ± 9	−	++	−
Average volume (μm^3)	260 ± 19	302 ± 14	456 ± 33	814 ± 25*	687 ± 99*	++	++	++
Average surface area (μm^2)								
With microvilli	144 ± 18	185 ± 21	258 ± 22	448 ± 32*	341 ± 48	++	++	++
Without microvilli	85 ± 15	125 ± 11*	134 ± 10	193 ± 15*	181 ± 25	++	++	−
Interstitial fibroblast								
Number (/mm^2)	677 ± 39	676 ± 57	284 ± 23	279 ± 7	254 ± 13	−	++	−
Average volume (μm^3)	442 ± 38	391 ± 16	607 ± 32	737 ± 29*	726 ± 44*	−	++	+
Interstitial macrophages								
Number (/mm^2)	43 ± 11	44 ± 6	14 ± 1	20 ± 3	20 ± 3	−	++	−
Average volume (μm^3)	26 ± 14	58 ± 37	109 ± 59	181 ± 34	147 ± 64	−	±	−
Endothelial cells								
Number (/mm^2)	1272 ± 72	1278 ± 60	592 ± 27	600 ± 14	525 ± 21	−	++	−
Average volume (μm^3)	258 ± 14	247 ± 17	413 ± 19	439 ± 10	483 ± 26*	−	++	+
Average surface area (μm^2)	794 ± 49	798 ± 30	1539 ± 75	1565 ± 39	1778 ± 59*	−	++	+
Alveolar macrophages								
Number (/mm^2)	49 ± 7	60 ± 10	20 ± 1	29 ± 3*	28 ± 2*	−	++	−
Average volume (μm^3)	622 ± 136	623 ± 95	1175 ± 221	1873 ± 127*	1546 ± 173	−	++	−

Note. All data are $\bar{x}$ ± SE. For the adult animals, comparisons among 3 groups were done by a multiple comparison procedure. *$p < 0.05$ for comparison to controls. †$p < 0.05$ for comparison to adult animals exposed to 0.5 ppm NO_2. Overall contrasts for significant effects due to age and/or to exposure to NO_2 include only those groups exposed to air or 0.5 ppm NO_2. ++$p < 0.01$, +$p < 0.05$, ±$p < 0.10$, −$p > 0.10$ for two-sided tests.

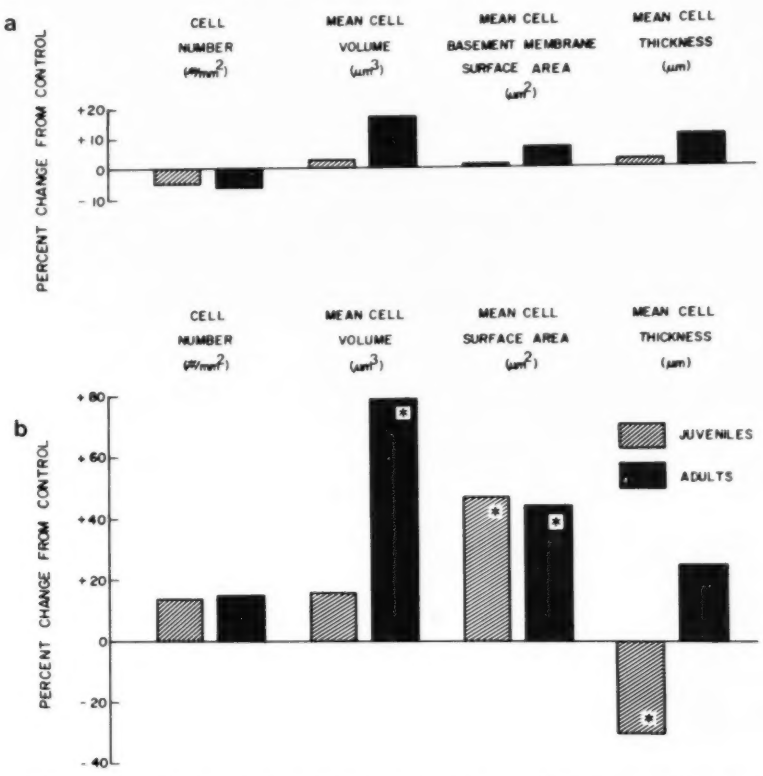


FIG. 2. The epithelium in the proximal alveolar region. The changes in the characteristics of type I and type II epithelial cells of juvenile and adult rats are expressed as percentage change from their respective control values after exposure to 0.5 ppm NO_2 for 6 weeks. (a) Type I epithelial cells. (b) Type II epithelial cells.

Changes in the alveolar epithelium. Exposure to 0.5 ppm NO_2 elicited a greater increase in total volume of type II alveolar epithelium than did 2.0 ppm NO_2. The higher concentration of NO_2, on the other hand, caused changes in the volume of both type I and type II epithelial cells. The total number and the mean size and surface area of type I cells were essentially unchanged by 0.5 ppm NO_2 (Fig. 5). Exposure to 2.0 ppm NO_2, in contrast, caused a moderate increase (17%) in the number of type I epithelial cells, a reduction (16%) in their mean surface area, and an increase (23%) in their mean thickness. Figures 6A and B contrast the normal type I epithelium with an area of thickened type I epithelium found after exposure to 2.0 ppm NO_2. Membrane blebbing of type I epithelium was commonly observed; areas of type I epithelium with frank disruption of the plasma membrane were found (Fig. 6C) at the higher dose of NO_2.

The number of type II epithelial cells was not significantly increased (Fig. 5). The mean cell size was drastically changed, being 79 and 51% larger in the 0.5 and 2.0 ppm exposed rats, respectively. Type II epithelial cells also showed spreading to cover a larger average alveolar surface after exposure to both concentrations of NO_2. The appearance of the type II cells in the 2.0-ppm exposed rats is illustrated in Fig. 4C. In the control rats, 98% of the proximal alveolar surface was covered by type I epithelial cells and approximately 2% by type II epithelium (Table 1). After exposure

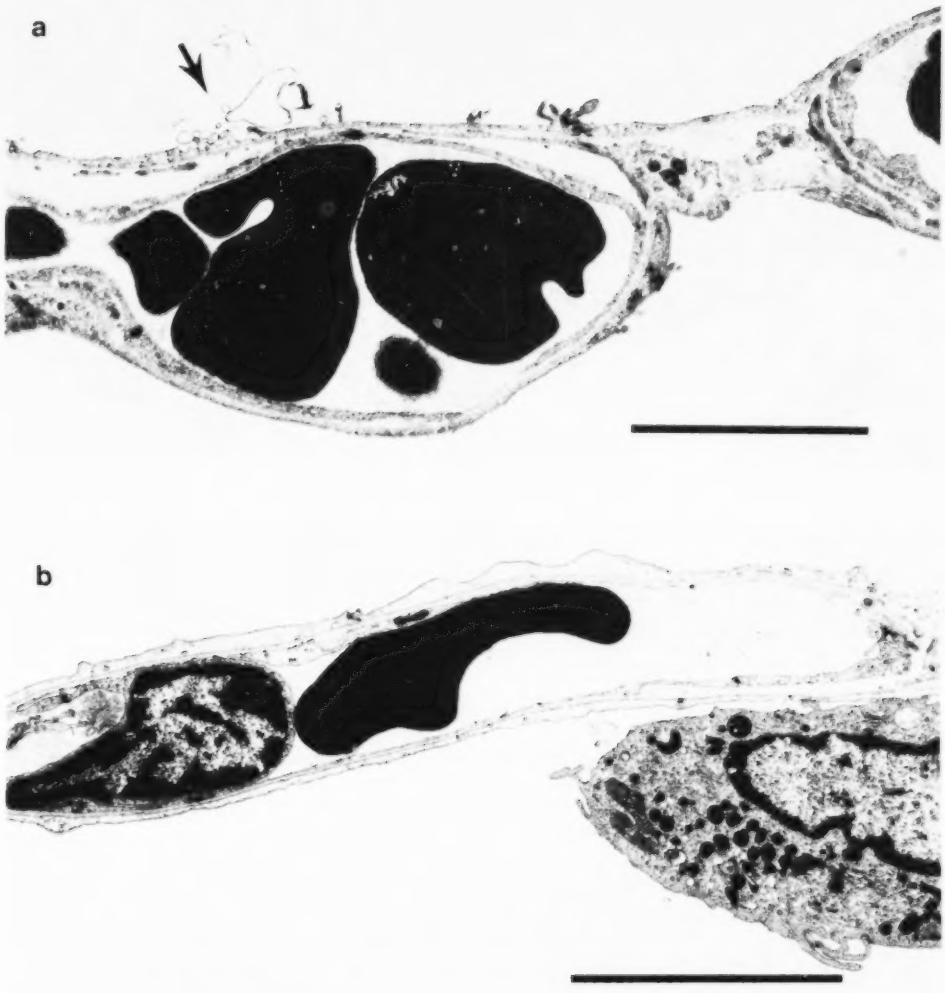


FIG. 3. Injury to type I epithelial cells in the proximal alveolar region after exposure to 0.5 ppm NO_2 for 6 weeks. (a) Type I epithelium from an adult rat. Membrane blebbing (arrow) was observed frequently. (b) Injured type I epithelium in an adult animal showing swelling and an electron lucent cytoplasm with few recognizable organelles. Bars = 5 μm.

to 0.5 and 2.0 ppm NO_2, the proportion of alveolar surface covered by type II epithelium increased to approximately 3%.

Changes in the alveolar interstitium. Tissue responses in the interstitium of adult rats exposed to 0.5 ppm NO_2 were similar in magnitude with those caused by 2.0 ppm NO_2. Fibroblasts were the major responding cells. Like type II epithelial cells, the fibroblasts became bigger (20%) as a result of NO_2 exposure, while their numbers were not altered. The amount of interstitial matrix was 20% greater

after exposure to 0.5 ppm NO_2 and 16% greater after exposure to 2.0 ppm NO_2.

Changes in alveolar macrophages. Both concentrations of NO_2 caused an increase in the volume of alveolar macrophages present in the proximal alveolar region of adult rats. Both the number and the mean cell volume of alveolar macrophages were increased. The alveolar macrohages in exposed animals contained increased numbers of vacuoles and lysosomes in their cytoplasm.

DISCUSSION

Morphometric data documenting alterations occurring in the proximal alveolar region following prolonged exposure to low concentrations of NO_2 are presented in this study. This region of the lung, along with the terminal and respiratory bronchioles, has been previously identified as the primary site of injury by oxidant gases (Stephens *et al.*, 1971, 1972). Fenestration, blebbing, and disruption of the plasma membrane of type I epithelial cells were the most commonly observed aberrations in the proximal alveolar region. Type II epithelial cells were found to be resistant to NO_2 and were induced to proliferate and differentiate during the process of epithelial repair (Evans *et al.*, 1972, 1975). Qualitative evaluations of these structural alterations have been described in a number of studies at both high (15–30 ppm) and low (0.5–2.0 ppm) concentrations of NO_2 (Freeman *et al.*, 1972; Stephens *et al.*, 1972).

In the current study we used specifically oriented sections through the proximal alveolar region to quantify the effects of NO_2 on both juvenile and adult animals. Minimal injury was found with rats exposed to 0.5 ppm NO_2 since 1 day of age. Adult rats, on the other hand, exhibited a range of tissue injuries to 0.5 ppm NO_2 and appeared to be more susceptible to NO_2 toxicity. Studies of newborn rats have shown that nursing pups are inherently resistant to both NO_2 and O_3 prior to weaning. Stephens *et al.* (1978, 1982) reported that susceptibility to oxidant gases increases immediately after weaning. It was postulated that the change of diet from high fat to high carbohydrate food may be an important factor (Hahn, 1979). Another factor in the lesser responsiveness of juvenile rats may be that the developing animals were in a growing phase. Stephens *et al.* (1982) have suggested that young cells are more resistant to oxidant injury. In addition, type II cells have been postulated to be more resistant to NO_2 challenge (Evans *et al.*, 1972, 1975; Blank *et al.*, 1982). We found that the ratio of type II to type I cells in the proximal alveolar region was 2.23 for the juvenile rats and 1.42 for the adult rats. Type I cells in the juvenile rats appeared to be smaller and covered less alveolar surface per cell. In other words, the epithelial cell population in the juvenile animals was made up of more type II cells which appear to be inherently resistant to NO_2 toxicity and type I epithelial cells which were not fully matured and therefore may be less susceptible to NO_2 than their counterpart in adult animals. A similar response in terms of finding increased epithelial cell tolerance to injury after exposure to an oxidant gas (O_3) has been previously reported (Evans *et al.*, 1985).

The surface density of basement membrane in the proximal alveolar region of exposed adult rats was found to have increased after exposure to NO_2. It is not clear what caused this change in basement membrane surface density. The lungs of the exposed adult rats were apparently well inflated, having total fixed lung volumes slightly larger than their matched controls. It is possible that septal injury in the proximal alveolar region caused a localized decrease in tissue compliance and consequently insufficient inflation of the proximal alveolar tissue. As was pointed out under Methods, variation in the degree of inflation can be compensated for by normalizing data to the surface density of alveolar basement membrane. Basement membrane surface area is a relatively stable reference point under conditions of mild lung injury. It will, under most conditions, change proportionally

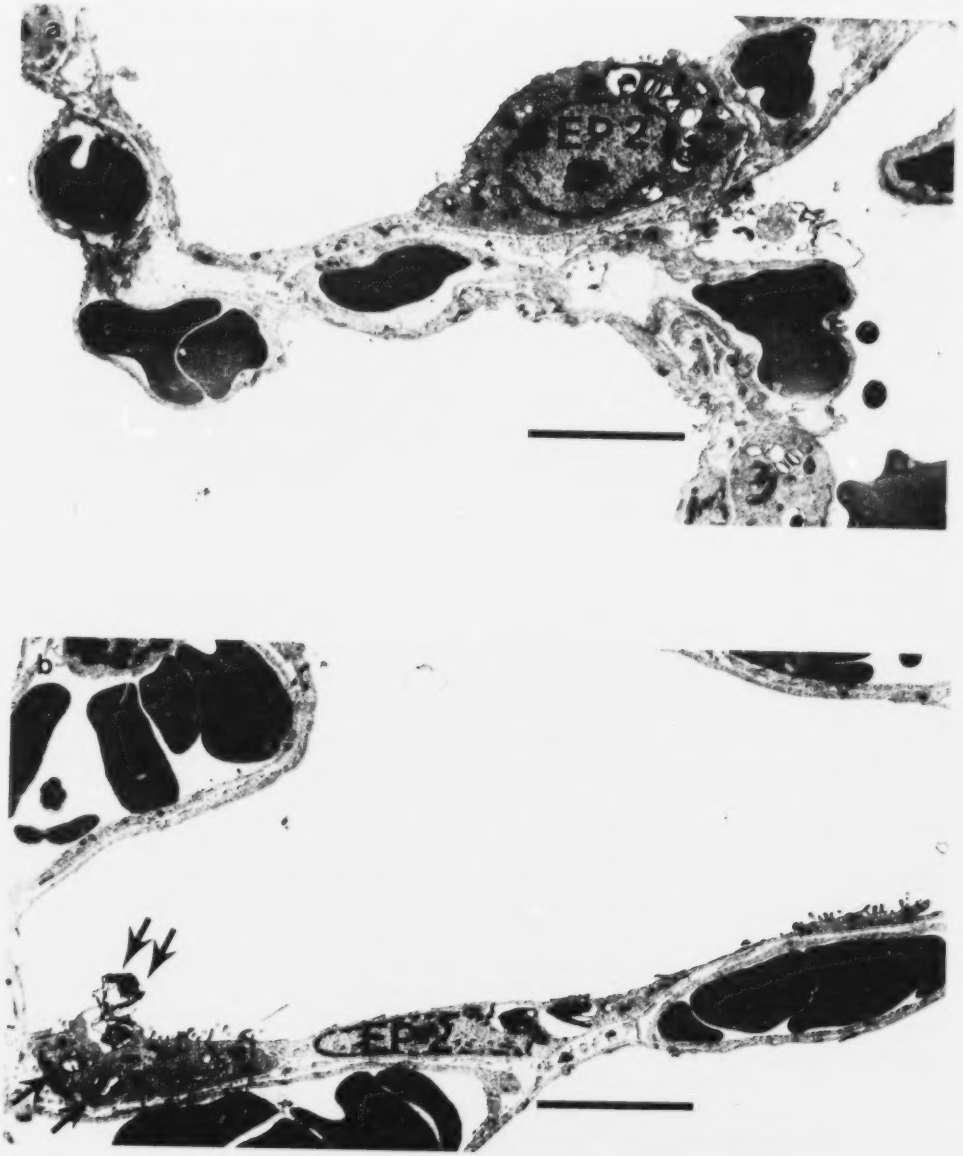


FIG. 4. Morphological changes found with type II epithelial cells (EP2) in juvenile and adult rats exposed to 0.5 ppm NO_2 for 6 weeks. (a) Normal type II epithelial cells representative of those found in both juvenile and adult animals. The shape of the cells was generally cuboidal. (b) Elongated and thinned type II epithelial cells in an exposed juvenile rat. The cells spread to cover a larger area. Lamellar bodies (arrows) could be found in these attenuated type II cells. One lamellar body (double arrow) was discharging its contents. (c) Hypertrophic type II epithelial cells found in an exposed adult rat. A contractile fibroblast (F) was seen in the interstitium. Bars = 5 μm.

FIG. 4—*Continued.*

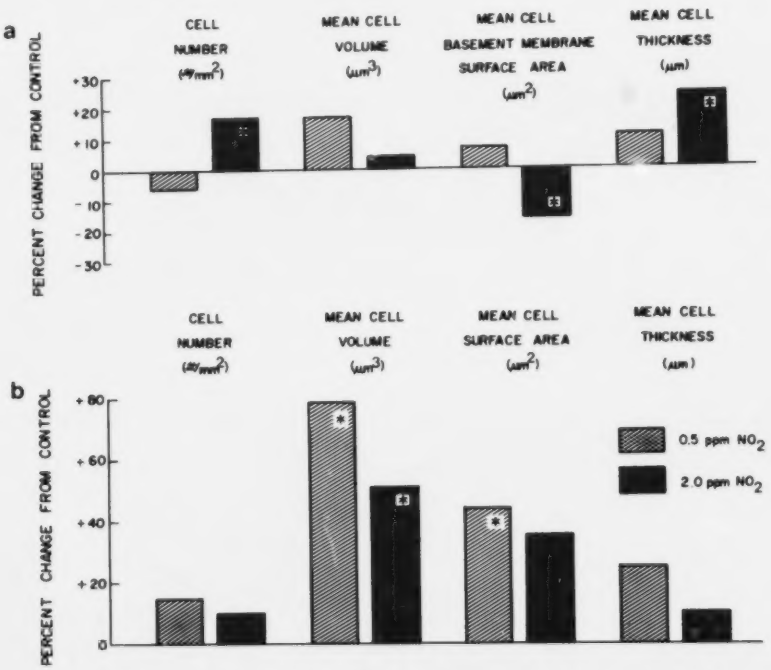
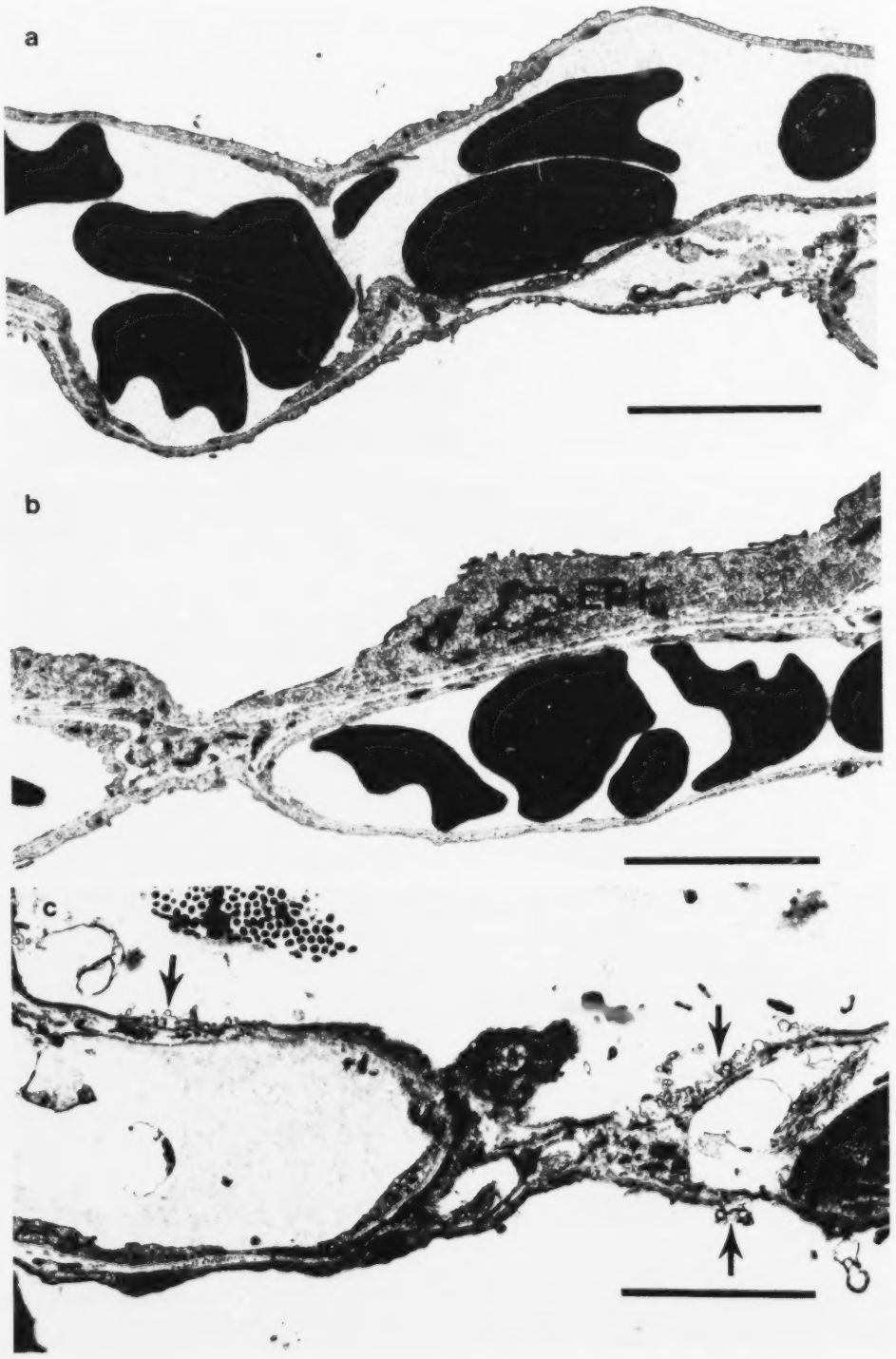


FIG. 5. The epithelium of the proximal alveolar region. The changes in the characteristics of type I and type II epithelial cells of adult rats are expressed as percentage of change from control values after exposure to 0.5 and 2.0 ppm NO_2 for 6 weeks. (a) Type I epithelial cells. (b) Type II epithelial cells.


a
b
EPI
c

with the degree of inflation. Since basement membrane surface density increased in the adult exposed animals, normalization against this parameter had the effect of reducing each of the observed experimental effects.

The changing patterns of reaction of the alveolar epithelial cells in the various groups of exposed animals reported in this study make it difficult to recognize the concentration dependency of the reaction. While type I cells are a major target for injury, repair appears to be sufficiently rapid to maintain the type I epithelium at normal levels until a significant oxidant stress is reached. Type II cells, as progenitor cells in the repair process, show much earlier evidence of reaction. After evaluating the pattern of changes in type II cell characteristics, we concluded that a consistent concentration-dependent pattern of injury was demonstrated by the three groups of exposed animals in this study. Depending on the nature and/or severity of injury to the alveolar epithelium, type II cells can respond in a number of ways. These include cell spreading, cell hypertrophy, and cell division and differentiation. When loss of type I epithelial cells is minimal, the first reaction of the type II epithelium may be simply to spread to cover the vacated alveolar surface. This process can be a smooth transition and no obvious injury of type I epithelium may be detected. As injury intensifies, further stress on type II cells may then result in overt cell hypertrophy. The alveolar type I epithelial cells are on the average 4 times larger in mass and cover 48 times more surface than type II cells. Thus spreading and hypertrophy of type II cells in combination with low rates of cell division and differentiation cannot be expected to keep up with a substantial degree of type I cell loss. If the rate of injury becomes high enough, more rapid type II cell proliferation and differentiation must ensue.

The changes in epithelial cell characteristics are organized in Fig. 7 to illustrate a rational sequence for type II cell reactions. The juvenile rats demonstrate the least evidence of epithelial injury. The only detectable sign of tissue reaction was a marked type II cell spreading and mild type II cell hypertrophy represented by increases in the mean cell surface area and total volume of type II epithelial cells, respectively. The effect of type II cell proliferation and differentiation in mild epithelial injuries may be indicated by the number of type I cells since recently differentiated type I cells will be smaller and cover less surface area than will fully mature type I cells. Since more young type I cells are required to cover the same amount of surface, the number of type I cells may be used as a crude index of the differentiation reaction going on in type II cells. Autoradiographic studies will be necessary to definitively address this issue. Panel C of Fig. 7 shows that there was no overt evidence of cell proliferation in the juvenile rats. Because type II cell spreading exceeded the degree of type II cell hypertrophy, the arithmetic mean thickness (volume to surface area ratio) of the type II cells in the juvenile rats decreased. In the adult animals exposed to 0.5 ppm NO_2, the combined effect of type II cell spreading and a much greater degree of type II cell hypertrophy resulted in thicker type II epithelial cells. However, a sufficient proliferative response to increase the net number of type I cells was still lacking. Rats exposed to 2.0 ppm NO_2 displayed all three types of type II cell reaction. Type II cell spreading and cell hypertrophy were present, and in addition there was a significant increase in the number of type I cells. The average type I cell was thicker

FIG. 6. Injuries to type I epithelium in the proximal alveolar regions of adult rats exposed to 2.0 ppm NO_2 for 6 weeks. (a) Normal type I epithelium from a control rat. (b) After exposure to 2.0 ppm NO_2, some type I epithelial cells (EP1) became thickened. Note that the epithelium on the lower side of the septum appears normal. (c) Extensive cell injury to a type I epithelial cell showing disruption of plasma membrane (arrows). Note cell debris in the air space. Bars = 5 μm.

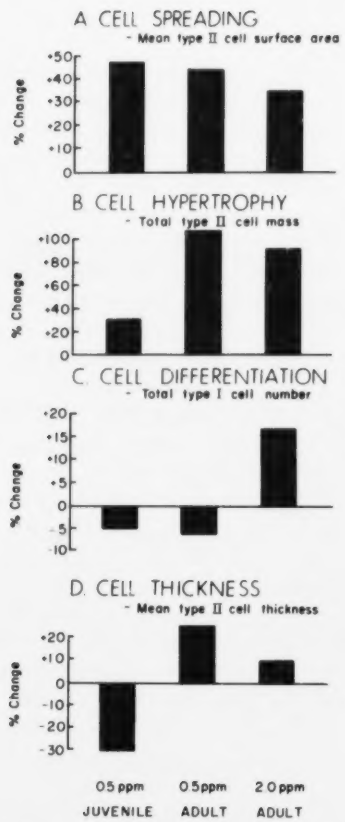


FIG. 7. Suggested patterns for the reactions of the alveolar epithelium to increasing degrees of injury caused by NO_2. The primary types of type II epithelial cell reaction are illustrated as: (a) cell spreading—expressed as mean type II cell surface area; (b) cell hypertrophy—expressed as mean type II cell volume; and (c) cell division and differentiation—indicated by changes in the population of product cells (type I). (d) Gives mean cell thickness which is the ratio of cell volume (hypertrophy) to cell surface area (spreading). The widely different responses in cell thickness can be explained by following the patterns of reactions in (a–c).

than normal and covered less average surface area, suggesting that they are younger and not yet fully differentiated. Average type II cell thickness in these animals returned toward normal, perhaps because the hypertrophy response was being counterbalanced by higher rates of cell division. Hypertrophy of type II epithelial cells could also be the result of direct interaction of these cells with NO_2 (Sherwin *et al.*, 1985). Qualitative evaluation of the exposed type II cells showed that the subcellular characteristics were normal.

In conclusion, we have demonstrated that exposure to low concentrations of NO_2 inflict injury to the alveolar epithelium. Evidence of type I cell injury was first indicated by changes in its precursor, the type II cells. Direct structural alterations in type I cells were found in adult animals exposed to NO_2. Adult rats were found to be more susceptible than juvenile rats to low concentrations of NO_2.

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